Annex G2

ICCVAM/NICEATM BG1Luc4E2 ER TA – Quantitative versus Qualitative Assessment of Cell Viability

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Supplemental Information for use in Discussing
Quantitative versus Qualitative Assessment of Cell Viability in the
BG1LUC4E2 ER TA Bioassay

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As part of the BG1LUC4E2 ER TA Bioassay protocol validation study, Xenobiotic Detection Systems, Inc. (XDS) evaluated the use Promega Corporation's CellTiter-Glo® quantitative cell viability assay. The assay measures cell viability based on the generation of luminescence signal proportional to the amount of ATP in viable cells. The CellTiter-Glo®assay requires the use of parallel plates as the luminescence signal interferes with the assessment of agonist or antagonist activity in the BG1LUC4E2 ER TA assay. The CellTiter-Glo® assay was conducted for all agonist and antagonist experiments during the BG1LUC4E2 ER TA assay protocol validation study. A qualitative method of assessing cell viability using visual observation previously developed by XDS was also conducted for all agonist and antagonist experiments during the BG1LUC4E2 ER TA assay protocol validation study. Criteria for assessing and scoring cell viability using XDS's visual observation method is provided in **Table 1**.

Table 1 Visual Observation Scoring Table

Viability Score	Brief Description[1]
1	Normal Cell Morphology and Cell Density
2	Altered Cell Morphology and/or Small Gaps between Cells
3	Altered Cell Morphology and/or Large Gaps between Cells
4	Few (or no) Visible Cells
1P	Score of 1 with Precipitate
2P	Score of 2 with Precipitate
3P	Score of 3 with Precipitate
4P	Score of 4 with Precipitate
5P	Unable to View Cells Due to Precipitate

A critical consideration in the conduct of the BG1LUC4E2 ER TA international validation study and the further standardization of the test method is the efficacy of limiting the assessment of cell viability to visual observation. This would greatly reduce the effort and cost of cell viability assessment by eliminating the need for running concurrent parallel plates required when using the CellTiter-Glo® method.

An initial examination of Raloxifene/E2 reference standard cell viability data using CellTiter-Glo® demonstrated that cell viability values of 80% or above did not correspond with a decrease in response in the BG1LUC4E2 ER TA assay. In general, CellTiter-Glo® values of 80% or above corresponded with a score of 1 in the visual observation method. Therefore, concentrations of test substance that caused a reduction in cell viability below 80% using CellTiter-Glo® or that had viability scores of 2 or more in the visual observation method were classified as cytotoxic and these data were not used to assess ER activity in the BG1LUC4E2 ER TA protocol standardization study.

In the protocol standardization study, CellTiter-Glo® results from the testing of eight substances covering a range of antagonist activities were compared to results from the XDS visual observation method (for discussion purposes, comparison of results is for

antagonist testing only because it is critical to distinguish whether reduction of luminescence is based on cytotoxicity or reduced ER mediated transcriptional activity).

In **Tables 2-6** below, selected results from five of the eight substances tested are provided as information to facilitate a discussion regarding the efficacy of limiting the assessment of cell viability to visual observation.

Table values highlighted in green indicate visual observation scores that did not correspond with CellTiter-Glo® % cell viability values (i.e., % cell viability of 80% or above should correspond to a visual observation score of 1).

Table values highlighted in blue indicate concentrations of substance that had acceptable cell viability as assessed by CellTiter-Glo® and would have been used to assess ER antagonist activity in the BG1LUC4E2 ER TA assay but would not have been used if assessment of cell viability was limited to visual observation.

"% Reduction of E2" is defined as the ability of a given concentration of test substance to reduce the ER TA activity induced by the E2 control (2.5×10^{-5} µg/mL, a concentration of E2 that induces 80-90% of maximum ER TA in the test system).

Butylbenzyl phthalate (**Table 2**) - classified as negative for antagonism in BRD.

4/12/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- Concentrations reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as negative for ER antagonist activity

4/15/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- No concentrations reducing E2 activity classified as cytotoxic, so used to assess ER activity
- Classified as positive for ER antagonist activity

4/18/06 experiment:

- CellTiter-Glo® values and visual observation scores do not correspond at concentrations of $2.50 \times 10^{+1}$ and $1.25 \times 10^{+1}$ µg/mL
- Concentrations reducing E2 activity classified as cytotoxic by either CellTiter-Glo® or visual observation, so not used to assess ER activity
- Classified as negative for ER antagonist activity but would have been classified as positive if using visual observations only

Table 2 Butylbenzyl phthalate

Date	Conc. µg/mL	% Reduction of E2	% Cell Viability	Visual Observation
4/12/06	$5.00 \times 10^{+1}$	67	76	2
	$2.50 \times 10^{+1}$	24	74	2
4/15/06	$5.00 \times 10^{+1}$	83	84	1
	$2.50 \times 10^{+1}$	68	82	1
	$1.25 \times 10^{+1}$	24	83	1
4/18/06	$5.00 \times 10^{+1}$	44	75	2
	$2.50 \times 10^{+1}$	35	70	1
	$1.25 \times 10^{+1}$	8	74	1

Flavone (**Table 3**) - classified as positive for antagonism in BRD (in all studies).

4/12/06 experiment:

- CellTiter-Glo® values and visual observation scores did not correspond
- Concentrations reducing E2 activity classified as cytotoxic by either CellTiter-Glo® or visual observation, so not used to assess ER activity
- Classified as negative for ER antagonist activity but would have been classified as positive if using visual observations only

4/15/06 experiment:

- CellTiter-Glo® values and visual observation scores do not correspond at 5.00 x 10^{+1} µg/mL
- Concentrations reducing E2 activity (2.50 x 10^{+1} and 1.25 x 10^{+1} µg/mL) not classified as cytotoxic , so used to assess ER activity
- Classified as positive for ER antagonist activity

4/18/06 experiment:

- CellTiter-Glo® values and visual observation scores do not correspond at concentrations of 2.50 x 10^{+1} and 1.25 x 10^{+1} µg/mL
- Concentrations reducing E2 activity classified as cytotoxic by either CellTiter-Glo® or visual observation, so not used to assess ER activity
- Classified as negative for ER antagonist activity but would have been classified as positive if using visual observations only

Table 3 Flavone

Date	Conc. µg/mL	% Reduction of E2	% Cell Viability	Visual Observation
4/12/06	5.00 x 10^{+1}	93	83	2
	2.50 x 10^{+1}	72	78	1
	1.25 x 10^{+1}	38	78	1
	6.25 x 10^{+0}	9	85	1
4/15/06	5.00 x 10^{+1}	99	91	2
	2.50 x 10^{+1}	90	86	1
	1.25 x 10^{+1}	37	85	1
	6.25 x 10^{+0}	0	86	1
4/18/06	5.00 x 10^{+1}	77	74	2
	2.50 x 10^{+1}	66	75	1
	1.25 x 10^{+1}	16	79	1

Nonylphenol (**Table 4**) - classified as positive for antagonism in BRD (in only one study).

4/15/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- $1.25 \times 10^{+1}$ µg/mL concentration reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as positive for ER antagonist activity at $6.25 \times 10^{+0}$ µg/mL

4/20/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- Concentrations reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as negative for ER antagonist activity

5/01/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- $1.25 \times 10^{+1}$ µg/mL concentration reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as positive for ER antagonist activity at $6.25 \times 10^{+0}$ µg/mL

Table 4 Nonylphenol

Date	Conc. µg/mL	% Reduction of E2	% Cell Viability	Visual Observation
4/15/06	$1.25 \times 10^{+1}$	99	29	4
	$6.25 \times 10^{+0}$	44	82	1
4/20/06	$1.25 \times 10^{+1}$	99	29	3
	$6.25 \times 10^{+0}$	61	75	2
5/01/06	$1.25 \times 10^{+1}$	99	64	3
	$6.25 \times 10^{+0}$	34	84	1

Progesterone (**Table 5**) - classified as negative for antagonism in BRD.

4/15/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- Neither concentration reducing E2 activity classified as cytotoxic, so used to assess ER activity
- Classified as positive for ER antagonist activity

4/20/06 experiment:

- CellTiter-Glo® values and visual observation scores do not correspond at 1.25 x 10^{+1} µg/mL
- Concentrations reducing E2 activity (2.50 x 10^{+1} and 1.25 x 10^{+1} µg/mL) classified as cytotoxic, so not used to assess ER activity
- Demonstrates "borderline" ER antagonist activity at 6.25 x 10^{+0} µg/mL

5/01/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- Concentrations reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as negative for ER antagonist activity

Table 5 Progesterone

Date	Conc. µg/mL	% Reduction of E2	% Cell Viability	Visual Observation
4/15/06	1.25 x 10^{+1}	73	86	1
	6.25 x 10^{+0}	39	92	1
4/20/06	2.5 x 10^{+1}	99	62	2
	1.25 x 10^{+1}	61	72	1
	6.25 x 10^{+0}	20	93	1
5/01/06	2.5 x 10^{+1}	87	62	3
	1.25 x 10^{+1}	49	69	3

***o,p'*-DDT (Table 6)** - classified as positive for antagonism in BRD (for one study).

4/20/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- Concentrations reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as negative for ER antagonist activity

5/01/06 experiment:

- CellTiter-Glo® values and visual observation scores correspond
- Concentrations reducing E2 activity classified as cytotoxic, so not used to assess ER activity
- Classified as negative for ER antagonist activity

5/05/06 experiment:

- CellTiter-Glo® values and visual observation scores do not correspond at 1.25 x 10^{+1} µg/mL
- Concentrations reducing E2 activity classified as cytotoxic by visual observation but not at 1.25 x 10^{+1} µg/mL with CellTiter-Glo®
- Classified as negative for ER antagonist activity when using visual observations only but would have been classified positive for antagonism at 1.25 x 10^{+1} µg/mL with CellTiter-Glo®

Table 6 *o,p'*-DDT

Date	Conc. µg/mL	% Reduction of E2	% Cell Viability	Visual Observation
4/20/06	5.00 x 10^{+1}	99	19	4
	2.50 x 10^{+1}	99	45	4
	1.25 x 10^{+1}	40	75	2
5/1/06	5.00 x 10^{+1}	99	26	4
	2.50 x 10^{+1}	99	59	4
	1.25 x 10^{+1}	22	74	2
5/5/06	5.00 x 10^{+1}	99	20	4
	2.50 x 10^{+1}	87	60	3
	1.25 x 10^{+1}	29	82	2